Phagocytosis of mast cell granule remnant-bound LDL by smooth muscle cells of synthetic phenotype: a scavenger receptor-mediated process that effectively stimulates cytoplasmic cholesteryl ester synthesis

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Abstract Mast cell granule remnants contain heparin proteoglycans and bind low density lipoproteins (LDL). Phagocytosis of such LDL-coated remnants by smooth muscle cells of synthetic phenotype (s-SMC) leads to cellular accumulation of LDL-derived cholesteryl esters (Wang et al. 1995. *Arterioscler. Thromb. Vasc. Biol.* **15:** 801–810). In the present study, we investigated the pathway by which granule remnants mediate the uptake of LDL by s-SMC and the effect of the remnants on the metabolism of LDL-derived cholesteryl esters in these cells. In vitro, the scavenger receptor ligands polyinosinic acid, acetylated LDL (AcLDL), and oxidized LDL (OxLDL) each inhibited the uptake of granule remnant-bound LDL maximally by 50–60%. When AcLDL and OxLDL were added as a mixture, uptake was totally inhibited. Conversely, the granule remnants inhibited the binding of AcLDL to s-SMC. We also found that granule remnants did not inhibit the lysosomal hydrolysis of LDL-derived cholesteryl esters in s-SMC. When s-SMC were incubated with LDL in the presence of granule remnants, the cellular contents of cholesteryl linoleate and cholesteryl oleate increased. These increases were retarded when an inhibitor of acyl-CoA:cholesterol acyltransferase (ACAT) was present, showing that the cholesteryl ester accumulation in the s-SMC was a cytoplasmic process due to reesterification of LDL-derived cholesterol and fatty acids. In summary, exocytosed mast cell granule remnants carry LDL into s-SMC by scavenger receptor-mediated phagocytosis, and induce formation of typical foam cells, filled with cytoplasmic cholesteryl ester droplets.—**Wang, Y., K. A. Lindstedt, and P. T. Kovanen.** Phagocytosis of mast cell granule remnant-bound LDL by smooth muscle cells of synthetic phenotype: a scavenger receptor-mediated process that effectively stimulates cytoplasmic cholesteryl ester synthesis. *J. Lipid Res.* 1996. **37:** 2155–2166.

Supplementary key words cholesteryl linoleate • cholesteryl oleate • heparin proteoglycans • lysosomes

In the atherosclerotic process, smooth muscle cells (SMC) in the arterial intima undergo structural and functional changes, one consequence of which is their conversion into foam cells (1–4). We have previously investigated the possibility that mast cells play a part in the conversion of rabbit aortic SMC of synthetic phenotype (s-SMC) into foam cells (5). In these studies, rat serosal mast cells were stimulated to exocytose their specific cytoplasmic organelles, the secretory granules. In the extracellular fluid, the soluble components of the granules, i.e., histamine, chondroitin sulfate proteoglycans, and a fraction of their heparin proteoglycans, become solubilized and are released from the granules. In contrast, two neutral proteases, chymase and carboxypeptidase A, and the bulk of the heparin proteoglycans remain tightly bound to each other, forming so-called "granule remnants" (6, 7). When such granule remnants were added to s-SMC cultured in medium enriched with low density lipoproteins (LDL), the granule remnants avidly bound LDL, and were then phagocytozed, inducing massive uptake of LDL by the rabbit aortic s-SMC. Ultimately, LDL-derived cholesteryl esters accumulated in the smooth muscle cells, with formation of foam cells.

The mechanism of granule remnant-mediated uptake of LDL by s-SMC and the intracellular metabolism of the LDL-derived cholesteryl esters in the cells were not investigated in the previous study (5). Inasmuch as the LDL particles were taken up when bound to the heparin

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; AcLDL, acetylated LDL, FCS, fetal calf serum; HPLC, high performance liquid chromatography; IFN-γ, interferon-γ; LDL, low density lipoproteins; LPDS, lipoprotein-deficient serum; mLDL, methylated LDL; OxLDL, oxidized LDL; PBS, Dulbecco's phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; s-SMC, smooth muscle cells of synthetic phenotype; TNF-α, tumor necrosis factor-α.

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proteoglycan-containing granule remnants, the possibility exists that it was the granule remnants that were recognized by the s-SMC, the LDL being passively carried into these cells. Indeed, Lindstedt, Kokkonen, and Kovanen (8) reported that a fraction of the granule heparin proteoglycans is released from the exocytosed granules, and that it is these soluble heparin proteoglycans that induce uptake of LDL by macrophages through scavenger receptor-mediated mechanisms. The scavenger receptors may also be important in the formation of SMC-derived foam cells, as SMC, when they appear in atherosclerotic lesions of rabbit aorta, express scavenger receptor activity (9). In addition, cultured medial SMC of rabbit aorta can be induced to express scavenger receptor mRNA by stimulation with phorbol esters (10), platelet secretion products (11), tumor necrosis factor-α (TNF-α), or interferon-γ (IFN-γ) (12). Bickel and Freeman (10) have cloned two cDNAs that encode type I and type II scavenger receptors isolated from rabbit aortic SMC of synthetic phenotype, and the protein sequences deduced from these isolates were found to be highly homologous to the scavenger receptors previously isolated from macrophages of other species, such as man, cow, and mouse. The above information led us to ask whether scavenger receptors are involved in the granule remnant-mediated uptake of LDL by s-SMC.

LDL, when complexed with heparin, fibronectin, and denatured collagen, is avidly taken up by mouse peritoneal macrophages (13). However, uptake of such complexes does not result in formation of typical foam cells with cytoplasmic cholesteryl ester droplets. Rather, the cells are filled with phagosomes containing material similar in appearance to the LDL-matrix complexes, revealing impaired degradation of the complexes. Because heparin-like polyanions have been reported to inhibit the fusion of phagosomes with lysosomes (14), the heparin of the complexes may have prevented lysosomal digestion of LDL. Furthermore, commercial heparin and other glycosaminoglycans have been reported to modify lysosomal function through formation of complexes with lysosomal enzymes, so inhibiting their digestive activity (15, 16). As heparin proteoglycans are major components of mast cell granule remnants, we also compared the ability of s-SMC to hydrolyze the cholesteryl esters contained in LDL when these particles are taken up by the cells either when free or when bound to mast cell granule remnants.

MATERIALS AND METHODS

Materials and animals

Sodium [^{125}I]iodide (13–17 mCi/μg) and [1α, 2α (n)-^{3}H]cholesteryl linoleate (30–60 Ci/mmol) were from Amersham International; Eagle's basal medium with Earle's salts (EBME) with 20 mM HEPES was from Flow Laboratories; RPMI 1640 culture medium, Dulbecco's phosphate-buffered saline (PBS), fetal calf serum (FCS), penicillin, streptomycin, and L-glutamine were from GIBCO; bovine serum albumin (BSA), compound 48/80, soybean trypsin inhibitor, collagenase type I A, trypsin, chloroquine diphosphate, latex beads, polyinosinic acid, cholesterol, cholesteryl oleate, and cholesteryl linoleate were from Sigma; "QuickPrep Micro mRNA Purification Kit", "First-strand cDNA Synthesis Kit", and rabbit scavenger receptor primers were from Pharmacia; AmpliTaq DNA polymerase was from Perkin Elmer. Cholesteryl ester transfer protein was a generous gift from Drs. C. Ehnholm and M. Jauhiainen, National Public Health Institute, Helsinki, Finland, and Sandoz compound 58-035 was obtained from Dr. J. Peter Slotte, Department of Biochemistry and Pharmacy, Abo Akademi, Turku, Finland. Male Wistar rats (300–500 g) and female New Zealand White rabbits (4–8 weeks) were purchased from the Laboratory Animal Center of the University of Helsinki.

Preparation of rabbit aortic smooth muscle cells

Aortic smooth muscle cells were isolated by carefully stripping pieces of the intima and media from the thoracic and upper parts of the abdominal aorta of 4- to 8-week-old female New Zealand white rabbits (5). The stripped segments were cut into 1-mm pieces, treated with 1 mg/ml of collagenase first for 1 h to remove the endothelial cells, washed with medium, and then dispersed with a mixture of collagenase (1 mg/ml) and elastase (0.5 mg/ml) in RPMI 1640 culture medium containing 12.5% of FCS. After incubation at 37°C for 2 h with occasional gentle agitation, the cell suspension was centrifuged at 800 *g* for 5 min. The cell pellet was washed and resuspended in medium A (RPMI 1640 culture medium containing 2 mM L-glutamine, 20% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin). The cells were inoculated at a density of 1×10^5 cells/ml and incubated in medium A and, at confluency, subcultured (1:2) for up to nine times. The SMC were used for the experiments when they had been subcultured at least five times.

Isolation of mast cell granule remnants and soluble heparin proteoglycans

Remnants of the cytoplasmic secretory granule and soluble heparin proteoglycans released from mast cell granules were isolated from the extracellular medium of stimulated rat serosal mast cells as described previously by Lindstedt et al. (8, 17). The concentration of granule remnants used in the experiments is expressed in terms of remnant protein, and that of the soluble heparin proteoglycans, in terms of their content of

Alcian Blue-reactive material with commercial heparin as standard.

Isolation, labeling, and modification of low density lipoproteins

Human low density lipoproteins (LDL; d 1.019–1.050) were isolated from plasma by sequential ultracentrifugation (18). [^{3}H]cholesteryl linoleate ([^{3}H]CL) was incorporated into LDL by incubating LDL with solid dispersions of [^{3}H]CL on celite, as described previously (19), except that cholesteryl ester transfer protein (activity: 5–10 μmol cholesteryl ester transferred/ml per h) and isolated LDL were used instead of full serum. The specific activity obtained varied from 20–60 dpm/ng LDL protein. LDL and [^{3}H]CL-LDL were reductively methylated ([^{3}H]CL-mLDL) by formaldehyde according to the method of Mahley et al. (20), acetylated ([^{3}H]CL-AcLDL) by repeated additions of acetic anhydride as described by Basu et al. (21), or oxidized ([^{3}H]OxLDL) by copper (incubated in 10 μM $CuSO_4$ at 37°C for 3 h) according to Hessler et al. (22). The concentrations of LDL, AcLDL, and OxLDL are expressed in terms of their protein concentrations.

Binding of mLDL, AcLDL, and OxLDL to granule remnants

The binding assays were conducted in 100 μl of medium B (EBME containing 10 mg/ml BSA, 1.2 mg/ml soybean trypsin inhibitor, 100 U/ml penicillin, and 100 μg/ml streptomycin) containing unlabeled mast cell granule remnants and [^{3}H]CL-mLDL, [^{3}H]CL-AcLDL, or [^{3}H]CL-OxLDL. The reaction mixtures were incubated at 0°C for 60 min. To determine the amounts of labeled lipoproteins bound to granule remnants, portions of the reaction mixtures were layered onto 300 μl of 5 mM Tris/HCl buffer containing 0.25 M sucrose and 10 mg/ml of BSA, pH 7.4. The tubes were then centrifuged at 12,000 *g* at 4°C for 10 min, and the supernatant of each tube was removed by aspiration. The granule remnant-containing pellets were resuspended in Optiphase Hisafe II scintillation fluid and counted for their ^{3}H radioactivity. The results are expressed as ng of lipoprotein protein bound per μg of granule remnant protein.

Binding and degradation of [^{125}I]AcLDL by s-SMC

Monolayers of s-SMC were prepared as described above, washed three times with PBS, and received 300 μl of medium C (EBME including 10 mg/ml BSA, 100 U/ml penicillin and 100 μg/ml streptomycin) containing the indicated concentrations of ^{125}I-labeled AcLDL with or without unlabeled AcLDL. The binding assay was conducted at 4°C for 3 h, and the degradation assay was conducted at 37°C for 16 h, as described by Goldstein and Brown (23).

Uptake by s-SMC of [^{3}H]CL-mLDL-coated granule remnants, [^{3}H]CL-AcLDL, and [^{3}H]CL-OxLDL

S-SMC monolayers were washed three times with PBS, and received 300 μl of medium C containing the indicated concentrations of either [^{3}H]CL-mLDL-coated granule remnants, [^{3}H]CL-AcLDL, or [^{3}H]CL-OxLDL. After incubation at 37°C for the indicated time periods, the medium was removed and the cells were further incubated at room temperature for 10 min in 500 μl of "washing buffer" (PBS supplemented with 5 mg/ml heparin, 10 mg/ml BSA, and 1 mg/ml soybean trypsin inhibitor). Finally, the cells were rinsed three times with PBS and collected into 500 μl of 0.2 M NaOH, and aliquots were removed for determination of both radioactivity and protein content. The results are expressed as uptake of μg mLDL, AcLDL, or OxLDL protein per mg cell protein.

Detection of scavenger receptor mRNA expression in s-SMC by RT-PCR

mRNA was extracted from rabbit s-SMC with "QuickPrep Micro mRNA Purification Kit", and used as a template (1 to 5 μg) to synthesize first-strand cDNA with a "First-strand cDNA Synthesis Kit" at 37°C for 1 h. PCR amplification of the cDNA synthesized was conducted in the following buffer: 10 mM Tris-HCl (pH 8.3 at 25°C), 50 mM KCl, 3.0 mM $MgCl_2$, 0.01% w/v gelatin, 250 nM each dNTP, 5 U AmpliTaq DNA polymerase, and 25 pmol of each primer. The sequences of the 5′ sense primer (5′AAGGAGCGTGTGCACAATGC3′) and the 3′ antisense primer (5′CTATCTCCTTTTTCTCCTGGG3′) were based on the sequence found in rabbit macrophage scavenger receptor cDNA (24). The reaction was carried out in a DNA thermal cycler with an initial 1-min denaturation step at 96°C, 40 cycles of PCR (94°C for 40 sec denaturation, 63°C for 60 sec annealing, and 72°C for 90 sec extension), followed by a final extension at 72°C for 5 min. The reaction mixture was analyzed by electrophoresis on a 2.5% agarose gel, and the specific 206-bp band was detected by staining with ethidium bromide. mRNA extracted from rabbit peritoneal macrophages was used as a positive control.

Quantitation of cellular content of free cholesterol and cholesteryl esters by HPLC

Cellular lipids were extracted in situ with hexane–isopropanol 3:2 (v/v) (25). The organic solvents were evaporated, and the phospholipids and fatty acids in the extracts were removed by elution with chloroform–isopropanol 2:1 (v/v) through a Bond Elut column (Sorbent: NH_2 aminopropyl). The eluted fractions were then evaporated to dryness with a stream of nitrogen, the residues were dissolved in acetonitrile–isopropanol 3:7 (v/v), and aliquots were taken for measurement of free cholesterol and cholesteryl esters by HPLC with a Spher-

isorb S5 ODS2 column, using acetonitrile–isopropanol 3:7 (v/v) as the mobile phase. The column was eluted at room temperature (20–24°C) at a flow rate of 300 μl/min and elution was monitored at 210 nm. Data were recorded and calculated on a HP 3396A integrator. Commercial free cholesterol, cholesteryl oleate, and cholesteryl linoleate were dissolved in the elution solvent as external standards, and cholesteryl heptadecanoate was used as internal standard (26). The results are expressed as μg cholesteryl oleate, or cholesteryl linoleate per mg cell protein.

Preparation of a lysosomal extract from rabbit aortic s-SMC

Aortic s-SMC were prepared as described above. When the cells were confluent, they were washed three times with cold PBS, harvested, and suspended in 250 mM cold sucrose containing 5 mM Tris-HCl buffer, pH 7.4. After sonication twice at 20 W for 20 sec and homogenization with a Teflon/glass homogenizer (both operations in an ice bath), the suspensions were centrifuged at 1000 *g* for 10 min at 4°C to remove cell debris. The supernatant was collected and centrifuged at 10,000 *g* for 45 min at 4°C to sediment the lysosome-rich fraction, as described by Maor and Aviram (27). The lysosome-rich pellets were resuspended in 150 mM NaCl, freeze-thawed four times to disrupt the lysosomes, and stored at -70°C until use. The lysosome-rich fraction was identified by assaying the activity of acid phosphatase. The activity of this enzyme was 10- to 17-fold higher in the lysosome-rich fraction than in the lysosome-free supernatant.

Hydrolysis of LDL-derived [^{3}H]cholesteryl linoleate by lysosomal extract

Hydrolysis of LDL-derived [^{3}H]cholesteryl linoleate by lysosomal extract was conducted either in 250 mM sodium acetate, pH 4.0, or in 250 mM Tris-HCl buffer, pH 9.0, to block lysosomal activity. After incubation at 37°C for 5 h, the lipids were extracted with chloroform–methanol 2:1 (v/v), and the content of free [^{3}H]cholesterol formed was measured by determining its radioactivity after separating it from other cellular lipids by thin-layer chromatography (28). In the control assay the lysosomal extract was omitted. The rate of hydrolysis of LDL-derived [^{3}H]cholesteryl linoleate by lysosomal extract is expressed as pmol free [^{3}H]cholesterol formed per μg protein in the lysosomal extract.

Lysosomal hydrolysis of LDL-derived [^{3}H]cholesteryl linoleate in s-SMC

The lysosomal compartment of s-SMC was enriched with LDL-derived [^{3}H]cholesteryl linoleate by inhibiting lysosomal degradation of LDL with chloroquine (29). For this purpose, the monolayers were incubated (in the absence of granule remnants) at 37°C in medium C containing 100 μg/ml of [^{3}H]CL-LDL and 100 μM chloroquine diphosphate. After incubation, the cells were incubated in the washing buffer for 10 min at room temperature, and then rinsed three times with PBS. Hydrolysis of cholesteryl esters in these cells was induced by incubating the cells at 37°C for 24 h in a medium containing 5% lipoprotein-deficient serum (LPDS) in the absence or presence of granule remnants with or without Sandoz 58-035, a specific ACAT inhibitor (30). After the 24-h hydrolysis, the cells were washed as above, the cellular lipids were extracted in situ with hexane–isopropanol 3:2 (v/v) (25), and the remaining cellular [^{3}H]cholesteryl esters were measured by determining their radioactivity after separating them from other cellular lipids by thin-layer chromatography (28).

Hydrolysis of cholesteryl esters derived from granule remnant-bound LDL in s-SMC

The s-SMC were first enriched with cholesteryl esters by incubating them with granule remnant-bound LDL for 48 h (in the absence of chloroquine). Hydrolysis of the LDL-derived cholesteryl esters was then allowed to proceed for 24 h in the presence of ACAT inhibitor, as described above.

Other methods

The protein content of granule remnants, lipoproteins, or cells was determined by the procedure of Lowry et al. (31), with bovine serum albumin as standard.

RESULTS

Mode of uptake of LDL-coated granule remnants by s-SMC

To demonstrate scavenger receptor expression in the s-SMC under our experimental conditions, we purified mRNA from the s-SMC, and detected scavenger receptor expression with RT-PCR, using sequences in rabbit macrophage scavenger receptor cDNA as primers. As shown in **Fig. 1A,** mRNA extracted from s-SMC expressed the same specific 206-bp band that was present in rabbit peritoneal macrophages. We also showed that ^{125}I-labeled acetylated LDL bound to the s-SMC with high affinity (Fig. 1B) and was degraded by the cells (Fig. 1C).

As we have shown previously using FITC avidin-coated granule remnants, s-SMC phagocytose granule remnants (diameters 0.5 to 1.0 μm) (5). When LDL was bound to granule remnants, LDL was also taken up, and LDL-derived cholesteryl esters accumulated in the cells. To investigate the pathway by which granule remnants of mast cells mediate the uptake of LDL by s-SMC, we

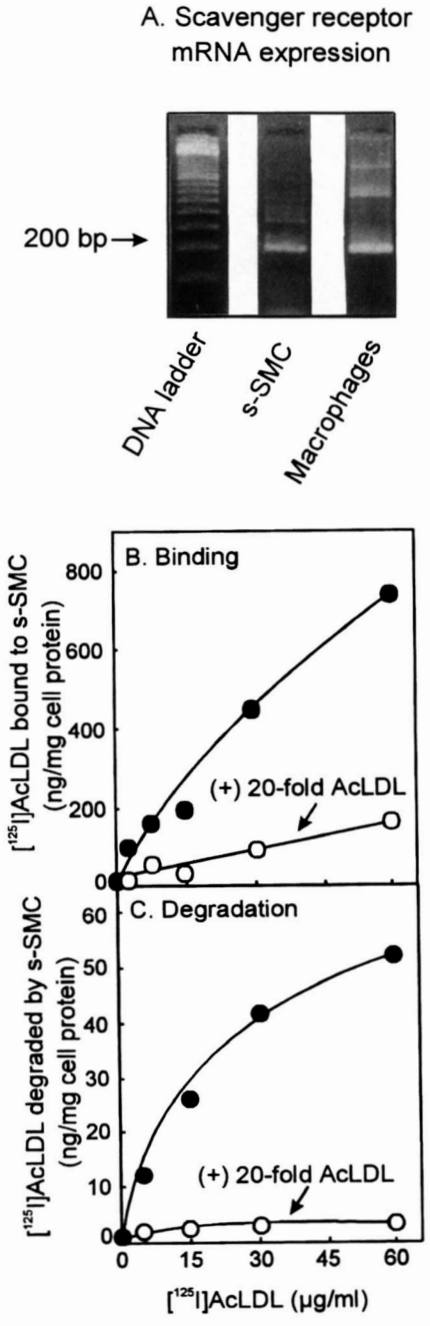

Fig. 1. Expression of scavenger receptor mRNA in s-SMC (A), and binding (B) and degradation (C) of [^{125}I]AcLDL by s-SMC. Panel A: scavenger receptor mRNA expression in s-SMC was detected by RT-PCR, using mRNA from rabbit macrophages as a positive control, as described in Materials and Methods. mRNA (1.3 μg) extracted from both cell types was used as template. Data shown are derived from one of four separate experiments. Panels B and C: monolayers of s-SMC were incubated in 300 μl of medium C containing the indicated concentrations of ^{125}I-labeled AcLDL in the absence or presence of unlabeled AcLDL either at 4°C for 3 h (binding assay; B) or at 37°C for 16 h (degradation assay; C), as described in Materials and Methods. Each value represents the average of duplicate incubations. Similar results were reproduced in another independent experiment.

used LDL in which the apoB-100 had been methylated and the cholesteryl linoleate had been labeled with tritium ([^{3}H]CL-mLDL). Such methylation totally prevents the uptake of free LDL by the s-SMC, but does not inhibit its binding to the remnants, and so allows studies to be conducted in which LDL is taken up by the cells only when bound to the granule remnants (5). To measure the rate of LDL uptake, it is necessary to label the LDL lipids (instead of LDL protein), as granule remnants proteolyze the apoB-100 of LDL in the extracellular medium (32). In the experiment shown in **Fig. 2,** we first incubated [^{3}H]CL-mLDL with unlabeled granule remnants to allow binding of the labeled mLDL to these remnants. In the complexes formed between mLDL and granule remnants, the molar ratio of LDL to granule remnants was about 8500:1 (in accord with previous results; (5, 33)), i.e., each granule remnant had bound, on average, 8500 LDL particles. When such [^{3}H]CL-mLDL-coated granule remnants were then added to cultures of s-SMC, 2.16 ± 0.98 μg of LDL protein/mg cell protein (100% values in Fig. 2) was taken up during a 6-h incubation. In the presence of different concentrations of polyinosinic acid, AcLDL, or OxLDL, all of which are known to bind to scavenger receptors (34, 35), uptake of the labeled mLDL was inhibited in a concentration-dependent fashion (Fig. 2; panels A–C). The insets in panels B and C show that neither AcLDL nor OxLDL bound to the granule remnants, and thus did not block the binding of mLDL to the remnants. Accordingly, the inhibitory effect of these modified lipoproteins on the uptake of granule remnant-bound mLDL by the s-SMC must have been due to their being scavenger receptor ligands (like polyinosinic acid) and occupying the cellular scavenger receptors. When both AcLDL and OxLDL were added to the incubation medium, uptake of granule remnant-bound [^{3}H]CL-mLDL was completely inhibited (**Fig. 3**). In a complementary experiment, we studied the effect of increasing concentrations of granule remnants on the binding of ^{125}I-labeled AcLDL to s-SMC, and found that the binding was inhibited by granule remnants in a concentration-dependent fashion (**Fig. 4**). However, the same quantities of latex particles (diameter 1.1 μm, i.e., within the size range of the granule remnants) failed to show any inhibitory effect on the binding of ^{125}I-labeled AcLDL to s-SMC. These observations are compatible with the idea that scavenger receptors of the s-SMC were involved in the phagocytotic uptake of the granule remnant-bound mLDL. Moreover, as the scavenger receptors do not recognize m-LDL, some component(s) of the remnants must have been responsible for recognition of the complexes by the receptors.

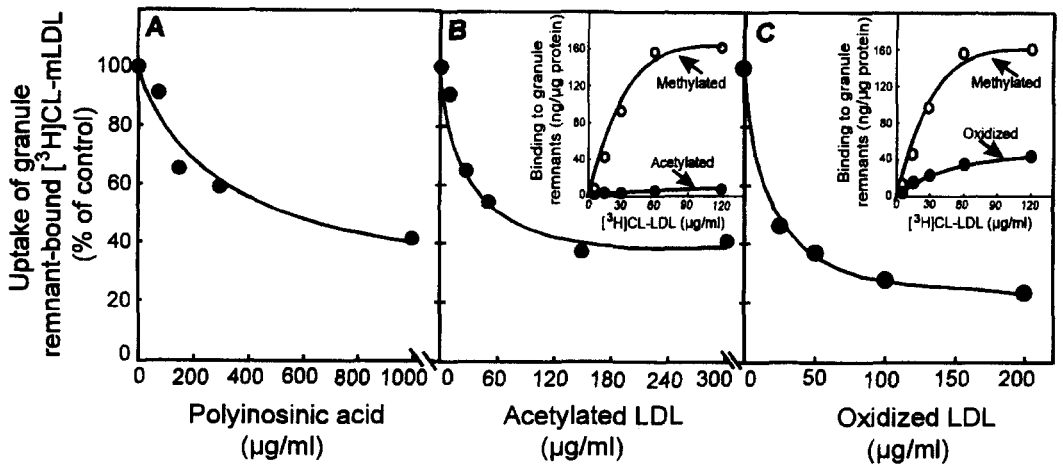

Fig. 2. Effect of increasing concentrations of polyinosinic acid (A), AcLDL (B), and OxLDL (C) on the ability of s-SMC to take up granule remnant-bound [^{3}H]CL-mLDL. Monolayers of s-SMC were incubated in the presence of increasing concentrations of the listed competitors in 300 μl of medium C containing 30 μg/ml of granule remnants and 60 μg/ml of [^{3}H]CL-mLDL, both of which had been preincubated at room temperature for 5 min prior to addition to the cultures. After incubation at 37°C for 6 h, the cells were collected and cellular uptake of [^{3}H]CL-mLDL was determined as described in Materials and Methods. The 100% value for the uptake of granule remnant-bound [^{3}H]CL-mLDL by the s-SMC in the absence of different competitors averaged 2.16 ± 0.98 μg of LDL protein/mg cell protein (A to C). The insets show the binding of [^{3}H]CL-AcLDL (panel B) and [^{3}H]CL-OxLDL (panel C) to granule remnants compared with [^{3}H]CL-mLDL. The binding assay was carried out in 100 μl of medium B containing 30 μg/ml of granule remnants and the indicated concentrations of each ^{3}H-labeled modified LDL, as described in Materials and Methods. Each value represents the average of triplicate incubations. Similar results were reproduced in another independent experiment.

Effect of granule remnants on intracellular metabolism of LDL-derived cholesteryl esters by s-SMC

The granule remnant-dependent uptake of LDL by the rabbit aortic s-SMC was previously found to result in a significant increase in the content of intracellular cholesteryl esters, with formation of foam cells (5). The next series of experiments in the current study were designed to specifically answer the question how granule remnants influence cholesteryl esters hydrolysis and reesterification in the s-SMC. In these experiments, we used native LDL instead of methylated LDL. Accordingly, during these incubations, a small fraction of the LDL could also be endocytosed through the pathway independent of granule remnants, reflecting more physiological conditions. As shown in **Fig. 5A,** when the cells were incubated in the presence of native LDL for up to 48 h, the cellular contents of cholesteryl oleate and cholesteryl linoleate progressively increased, the predominant ester being cholesteryl oleate, its content being 1.7 times higher than that of cholesteryl linoleate. If, however, the incubation medium contained granule remnants, in addition to LDL, the cellular content of cholesteryl esters increased more rapidly: the content of cholesteryl oleate was increased 7-fold, and that of cholesteryl linoleate over 20-fold after incubation for 48 h, rendering cholesteryl linoleate the predominant type of cholesteryl ester in the s-SMC. Accordingly, the cellular cholesteryl linoleate/cholesteryl oleate ratio increased more rapidly when granule remnants were also added to the incubation medium (Fig. 5B).

The predominant type of cholesteryl ester in the LDL particles is cholesteryl linoleate (the ratio of cholesteryl linoleate to cholesteryl oleate in our preparations was 4.2 ± 1.3; mean ± SD; n = 8). As noted above, the cholesteryl ester-filled s-SMC also contained more cholesteryl linoleate than cholesteryl oleate. This could have resulted from intracellular accumulation of some unhydrolyzed LDL, due, for instance, to inhibition of lysosomal enzyme activity. Indeed, heparin, when endocytosed by mouse peritoneal macrophages (13) or incubated in a cell-free system enriched with lysosomal extract (16), has been reported to inhibit lysosomal enzyme activity. To study the effect of granule remnants on lysosomal hydrolysis of cholesteryl esters, we measured, in the absence and presence of granule remnants, the rate of hydrolysis of LDL-derived [^{3}H]cholesteryl linoleate at pH 4 in a cell-free system enriched with lysosomal extract from the s-SMC. For comparison, we included in the system soluble heparin proteoglycans released from the mast cell granules, or low molecular weight (LMW), or high molecular weight (HMW) commercial heparins. As shown in **Fig. 6,** both LMW and HMW heparin inhibited the activity of lysosomal acid cholesteryl esterase by decreasing the rate of hydrolysis of [^{3}H]cholesteryl linoleate. In sharp contrast, mast cell granule remnants and the soluble heparin proteoglycans released from the granules failed to inhibit this

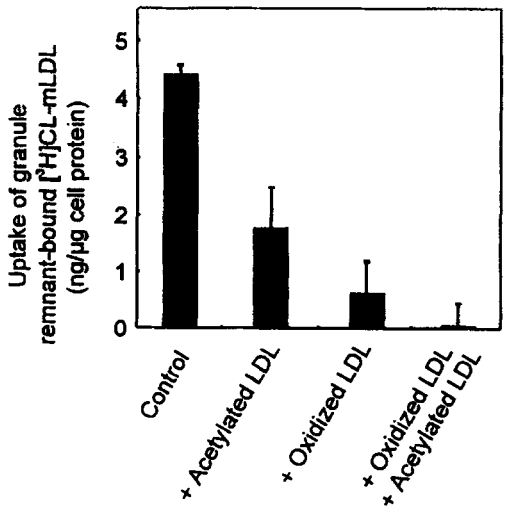

Fig. 3. Additive effect of AcLDL and OxLDL on the ability of s-SMC to take up granule remnant-bound [^{3}H]CL-mLDL. Monolayers of s-SMC were incubated in the presence of 150 μg/ml of either AcLDL alone, OxLDL alone, or a mixture of the two modified LDLs (150 μg/ml of each) in 300 μl of medium C containing 20 μg/ml of granule remnants and 60 μg/ml of [^{3}H]CL-mLDL, both of which had been preincubated at room temperature for 5 min prior to addition to the cells. After incubation at 37°C for 6 h, the cells were collected and cellular uptake of [^{3}H]CL-mLDL was determined, as described in Materials and Methods. Values are means ± SD of triplicate incubations.

lysosomal activity. To investigate whether granule remnants would affect lysosomal acid lipase activity in intact cells, we incubated s-SMC with [^{3}H]CL-LDL in the presence of chloroquine to induce lysosomal accumulation of LDL-derived cholesteryl esters. The cells were then washed and incubated in the presence of LPDS to induce efflux of cholesterol and net hydrolysis of cellular cholesteryl esters. During this incubation, half of the dishes received granule remnants. In a parallel experiment, we included an inhibitor of ACAT (Sandoz compound 58-035) to ensure that the [^{3}H]cholesterol formed did not become reesterified in the cytoplasm of the cells. It appeared that hydrolysis of the cholesteryl esters was not significantly influenced by the presence of granule remnants (**Table 1**). As chloroquine had inhibited lysosomal lipase activity by only 60% (as judged from the cellular content of unesterified labeled cholesterol at the end of the 24-h incubation period with chloroquine which was about 40% of the content of labeled cholesteryl esters), a minor fraction of the [^{3}H]cholesteryl esters probably resided in the cytoplasmic compartment of the cells, and was also hydrolyzed. Finally, in a complementary experiment, we omitted chloroquine to allow cholesteryl esters to accumulate mainly in the cytoplasmic compartment, and induced uptake of LDL through the scavenger receptor-mediated phagocytosis by adding granule remnants to the incubation medium. Cholesteryl esters were then allowed to accumulate by incubating the cells for 48 h with LDL and the remnants. During a subsequent 24-h incubation, we determined the magnitude of hydrolysis of the LDL-derived cholesteryl esters in the presence of compound 58-035 and granule remnants. As shown in **Fig. 7,** when reesterification of cytoplasmic cholesterol was inhibited by compound 58-035, both cellular LDL-derived cholesteryl oleate (panel A) and cholesteryl linoleate (panel B) were completely hydrolyzed. In summary, the above observations strongly suggest that lysosomal (and cytoplasmic) hydrolysis of LDL cholesteryl esters is not inhibited when LDL has entered the cellular lysosomal (phagosomal) compartment either alone or together with mast cell granule remnants.

We have previously reported that uptake of granule remnant-bound LDL induced the incorporation of albumin-bound [^{14}C]oleate into cholesteryl [^{14}C]oleate in s-SMC (5). To evaluate in greater detail the ability of the rabbit aortic s-SMC to esterify LDL-derived cholesterol with oleate and linoleate after granule remnant-bound LDL had been carried into the cells, we included in the incubation system albumin-bound oleate and linoleate separately in the presence or absence of compound 58-035. As shown in **Fig. 8A** (without compound 58-035), LDL, as compared with medium alone, produced slight increases in both cholesteryl oleate and linoleate (b vs. a). If the incubation medium contained granule remnants in addition to LDL, the content of cholesteryl linoleate was about twice that of cholesteryl oleate (c). Further, when albumin-bound oleate was added to the incubation mixture in the presence of LDL only, the predominant cellular cholesteryl ester was cholesteryl oleate (d), whereas, when albumin-bound linoleate was added instead of oleate, the preferential cholesteryl ester was cholesteryl linoleate (e). Similarly, when oleate was added to incubation medium containing both LDL and granule remnants, the content of cholesteryl oleate increased and equaled that of cholesteryl linoleate (f); when linoleate was added, the content of cholesteryl linoleate rose dramatically and became the predominant type of ester (90%) (g). Addition of the specific ACAT inhibitor blocked the granule remnant-mediated increases in cholesteryl esters in the absence (c) or presence of either oleate (f) or linoleate (g) (Fig. 8B). Thus, the cellular accumulation of LDL-derived cholesteryl esters depended on ACAT activity and, hence, must have involved both lysosomal hydrolysis and cytoplasmic reesterification of the esters. Moreover, the findings in rabbit aortic s-SMC demonstrated that ACAT, even though it preferentially esterifies oleate with cholesterol (25, 36, 37), will also esterify linoleate. Indeed, when both oleate and linoleate are supplied, which fatty acids are esterified is partly determined by

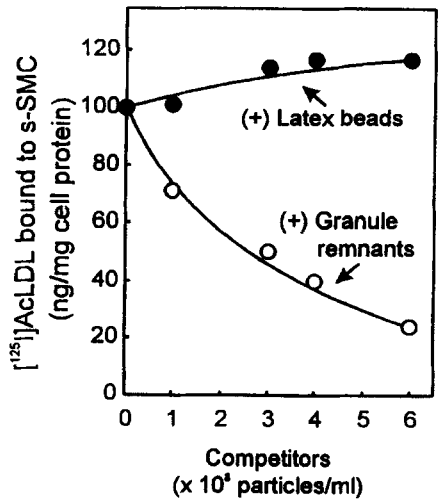

Fig. 4. Effect of increasing concentrations of granule remnants and of latex beads on the binding of [^{125}I]AcLDL to s-SMC. Monolayers of s-SMC were prepared, and binding of [^{125}I]AcLDL (10 μg/ml) to the s-SMC was conducted in the presence of the indicated concentrations of competitors at 4°C for 3 h, as described in Materials and Methods. Each value represents the average of duplicate incubations. A similar result was reproduced in another independent experiment.

the relative amounts of the two types available. Thus, when mast cell granule remnants carry LDL into s-SMC in a medium devoid of other sources of cholesteryl esters, and lysosomal hydrolysis of LDL is effective (four times more linoleate being released than oleate), the fatty acid composition of the cytoplasmic cholesteryl esters will tend to reflect that of the LDL particles carried into the cells.

DISCUSSION

In the present study we show that exocytosed cytoplasmic granules of rat serosal mast cells carry LDL into rabbit aortic s-SMC through scavenger receptor-mediated phagocytosis. Previous reports have shown that interaction of a high-affinity heparin subfraction (MW 17,000; from bovine lung) with LDL leads to scavenger receptor-mediated endocytosis of the lipoprotein and accumulation of cholesteryl esters in macrophages (38) and that lipoprotein-proteoglycan complexes isolated from human atherosclerotic lesions stimulate cholesteryl ester accumulation in human monocyte-derived macrophages with involvement of the scavenger receptor (39, 40). In analogy, heparin proteoglycans of mast cell granule remnants may be the component of the remnants that is responsible for the scavenger receptor-mediated uptake of granule remnant-bound LDL by the s-SMC. This uptake rapidly increases the content of cytoplasmic cholesteryl esters in the s-SMC. Studies on the mechanism of this accumulation with the aid of inhibitors of lysosomal enzymes and the cytoplasmic enzyme ACAT revealed that the cholesteryl esters of LDL had been hydrolyzed in lysosomes and reesterified in the cytoplasmic compartment of the cells. This sequence of events in cellular cholesterol metabolism is identical to that of the classical LDL receptor pathway (41). The similarity between LDL receptor-mediated endocytosis and scavenger receptor-mediated phagocytosis can be explained in terms of the fact that the LDL-containing endosomes and phagosomes ultimately fuse with the primary lysosomes, and so allow the lysosomal enzymes to act on the granule remnant-bound LDL.

The uninhibited cellular metabolism of LDL particles bound to heparin proteoglycans of mast cell granule remnants was an unexpected finding, as many polyanions, by means of their strong negative charges, have been shown to inhibit the various steps involved in the degradation of LDL. Thus, dextran sulfate and suramin

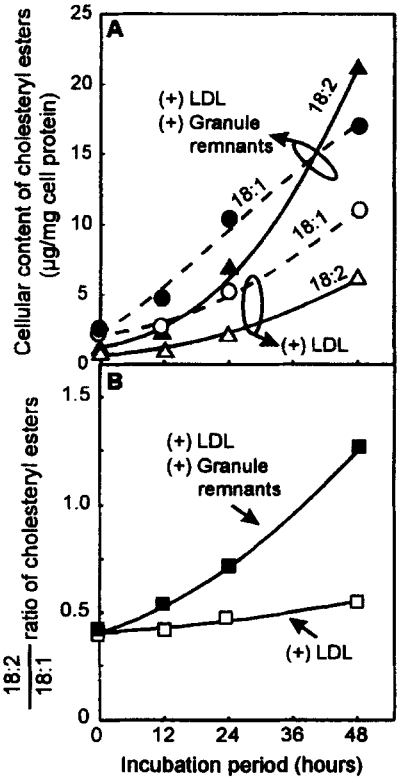

Fig. 5. Effect of granule remnants on the content of cholesteryl oleate (18:1) and linoleate (18:2) in s-SMC. Monolayers of s-SMC were incubated in 1 ml of medium C containing 100 μg/ml of LDL in the presence or absence of 5 μg/ml of granule remnants. After incubation at 37°C for the indicated time periods, the cellular lipids were extracted, and assayed for their cholesteryl ester content as described in Materials and Methods (panel A). Each value represents the mean of triplicate incubations. Similar results were reproduced in another independent experiment. In panel B, the ratio of cholesteryl linoleate/cholesteryl oleate has been calculated and plotted as a function of the incubation time.

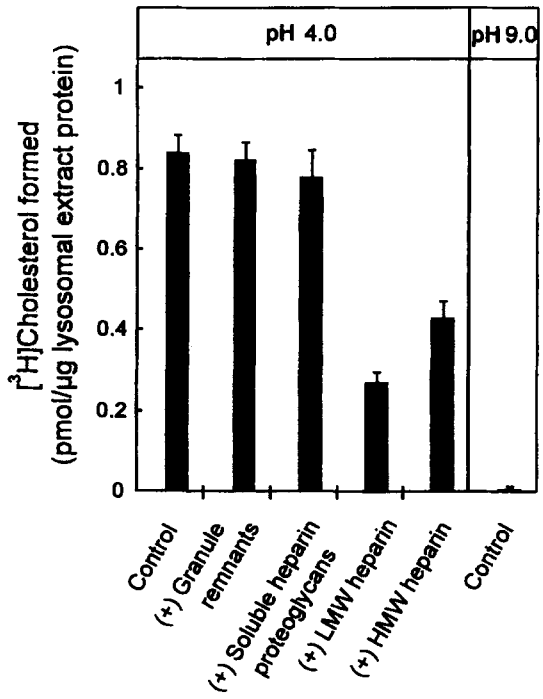

Fig. 6. Effect of granule remnants, soluble heparin proteoglycans released from granules, and two types of commercial heparin on the rate of hydrolysis of LDL cholesteryl linoleate ([^{3}H]CL-LDL) by a lysosomal extract of s-SMC. A lysosomal extract was prepared from s-SMC as described in Materials and Methods. A volume of extract protein equivalent to 150 μg and 5 μg [^{3}H]CL-LDL were incubated in 500 μl of 250 mM sodium acetate, pH 4.0, in the presence of either granule remnants, soluble heparin proteoglycan released from granules, low molecular weight (LMW) heparin, or high molecular weight (HMW) heparin. Each of the above added preparations contained 12.5 μg of Alcian blue-reactive material with HMW heparin as standard. After incubation at 37°C for 5 h, the content of [^{3}H]cholesterol formed was determined as described in Materials and Methods. A control incubation at pH 9.0 was carried out in 250 mM Tris-HCl (right). Values are means ± SD of triplicate incubations.

inhibit the fusion of phagosomes with lysosomes (14), and heparin and chondroitin sulfate inhibit the activity of lysosomal enzymes (15, 16). In consequence, substances normally degraded by lysosomal enzymes may escape digestion when coupled to a polyanion. Indeed, degradation of LDL particles has been shown to be impaired when the LDL was bound to complexes composed of heparin, fibronectin, and denatured collagen (13). Why the natural heparin proteoglycans of granule remnants acted differently from the related substances cited above, and did not inhibit the access of lysosomal enzymes to the contents of phagosomes or the activity of the lysosomal enzymes, is not known. It should be remembered, however, that the physiological fate of exocytosed granule remnants in tissues is phagocytosis, and ultimately complete degradation in the cells surrounding the degranulated mast cells (42, 43). Therefore, the ability to effectively degrade mast cell granule remnants is a normal cellular function that has evolved during the course of time. In contrast to the commercial polyanions, the "native heparin" in the granule remnants is in the form of large proteoglycan monomers (average MW 750,000), each containing about 10 heparin chains (average MW 75,000) attached to a core protein (44). This contrasts with most of the polyanions used in the studies cited above, which are single glycosaminoglycan chains of various lengths (MW ranging from 1500 to 500,000) without a core protein.

The typical foam cells in human atherosclerotic lesions contain cytoplasmic droplets in which the predominant type of lipids is cholesteryl oleate (37). This also applies to experimental models of atherogenesis, such as the subcutaneous carrageenan granuloma model in rabbits (45) and aortic atherosclerosis in cholesterol-fed rabbits (46). The predominance of oleate is probably due to the specificity of ACAT, which preferentially uses oleate as its substrate (36, 47). For this reason, most investigators, when studying LDL-dependent synthesis of cellular cholesteryl esters, have added labeled oleate-albumin complexes to cells (34). In our previous report, we also found that granule remnant-mediated uptake of LDL increases the incorporation of labeled oleate into cholesteryl esters in rabbit aortic s-SMC in vitro (5). However, when cellular lipid accumulation was now monitored by measuring the accumulation of cholesteryl linoleate and cholesteryl oleate, the main cholesteryl ester components of LDL, an increase was observed in cellular cholesteryl linoleate and, to a lesser extent, of cholesteryl oleate. Whether the lysosomal clearance of lipid is impaired (48) or not (this study), the preferential accumulation of cholesteryl linoleate in the cells taking up LDL in vitro is understandable, as the predominant component of cholesteryl esters in LDL is linoleic acid. Moreover, as shown by Slotte and Bierman (30) in human arterial SMC, the lysosomal acid choles-

TABLE 1. Effect of granule remnants on hydrolysis of LDL-derived [^{3}H]cholesteryl esters by s-SMC

Treatment	Cellular [^{3}H]Cholesteryl Esters	
	Sandoz 58-035	+ Sandoz 58-035
	pmol/mg cell protein	
Before hydrolysis	1156 ± 175	
After hydrolysis		
- granule remnants	569 ± 80	444 ± 170
+ granule remnants	498 ± 69	428 ± 142

Lysosomal accumulation of LDL in s-SMC was induced by incubating monolayers of the cells at 37°C for 24 h with 100 μg/ml of [^{3}H]CL-LDL in the presence of 100 μM chloroquine diphosphate (without granule remnants). The monolayers were then washed, and incubated for another 24 h in fresh medium containing 5% LPDS in the absence or presence of granule remnants (5 μg/mL) with or without Sandoz 58-035 (5 μg/ml). After incubation, the remaining cellular [^{3}H]cholesteryl esters were quantified, as described in "Materials and Methods". Values are means ± SD (n = 3) of results from three separate incubations.

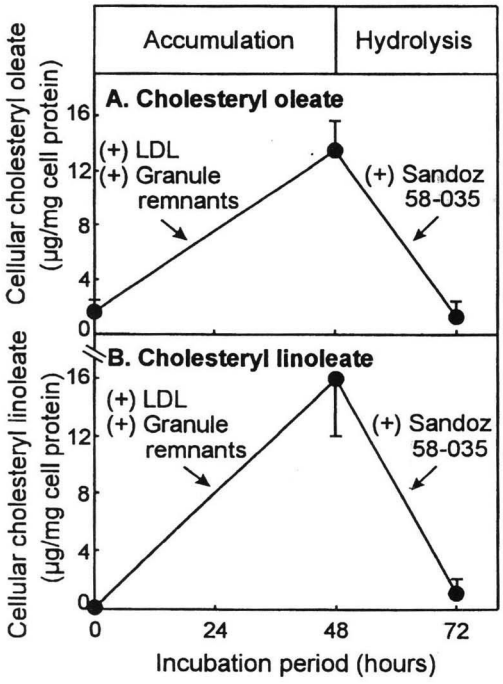

Fig. 7. Effect of granule remnants on accumulation and hydrolysis of cholesteryl oleate and cholesteryl linoleate in s-SMC. Monolayers of s-SMC were incubated at 37°C for 48 h in medium C containing 100 µg/ml of LDL and 5 µg/ml of granule remnants. The monolayers were then washed, the medium was replaced with fresh medium C containing 5% LPDS, 5 µg/ml of Sandoz compound 58-035, and 5 µg/ml of granule remnants, and incubation was continued at 37°C for another 24 h. The cellular contents of cholesteryl oleate and cholesteryl linoleate were determined at the start of incubation (at 0 h), after incubation with LDL and granule remnants for 48 h (at 48 h), and after incubation with LPDS and 58-035 for a further 24 h (at 72 h), as described in Materials and Methods. Values are means ± SD of five incubations from two separate experiments.

terol esterase preferentially hydrolyzes polyunsaturated cholesteryl esters, especially cholesteryl linoleate. Granule remnants did not inhibit the lysosomal esterase of the s-SMC. Hence, granule remnant-mediated uptake of LDL must have been followed by increased delivery of unesterified cholesterol, and of linoleate and oleate in roughly the same ratio as in native LDL (4.2 ± 1.3 in our preparations; n = 8), from lysosomes into cytoplasm, resulting in the fatty acid pattern of cytoplasmic cholesteryl esters that was observed. However, even when the s-SMC were incubated with granule remnant-bound LDL for 48 h (Fig. 5) or for 60 h (Fig. 8), the ratio of cholesteryl linoleate to cholesteryl oleate still did not reach the value observed in the native LDL (i.e., 1.3 and 2.0, respectively vs. 4.2). The notion that the s-SMC are able to esterify both linoleate and oleate was supported by the findings that addition to the incubation mixture of linoleate or oleate complexed with albumin stimulated an increase in the cellular content of cholesterol esterified with the respective fatty acid. In addition, we found (data not shown) that when human monocyte-derived macrophages or mouse peritoneal macrophages are incubated in vitro with AcLDL or with native LDL and granule remnants, more cholesteryl linoleate accumulates than cholesteryl oleate. Why, then, during atherosclerosis in vivo, is it cholesteryl oleate that accumulates in the foam cells (25, 37), and not cholesteryl linoleate? This difference between the observations in vitro and in vivo may be explained by the combined effects of the relative specificity of ACAT for the incorporation of oleate into cholesteryl esters (36, 47), and the continuous hydrolysis–reesterification cycle of cholesteryl esters in the cytoplasm of foam cells of the intima (25). Thus, over long periods of time, the oleate content of the cholesteryl ester particles would tend gradually to increase. The high cholesteryl oleate content of intimal foam cells may also be explained by the difference in fatty acid distribution between plasma LDL and aorta-derived LDL: in the LDL particles iso-

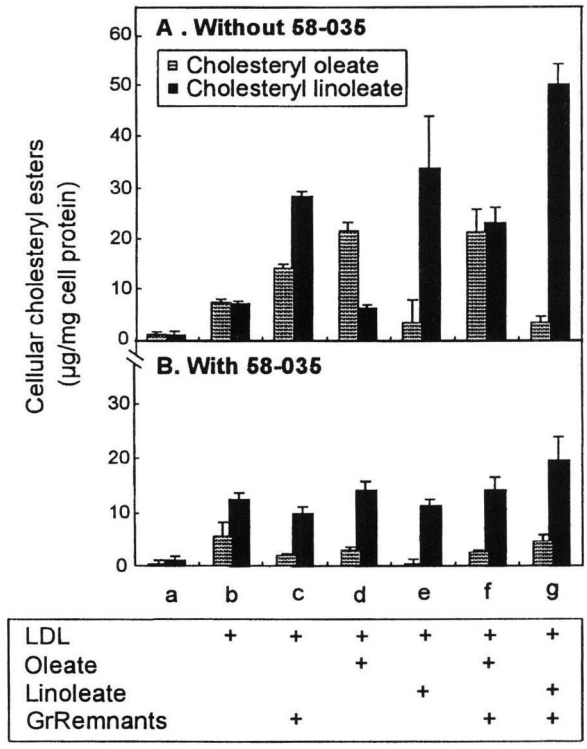

Fig. 8. Effect of albumin-bound oleate and linoleate on the fatty acid profile of cholesteryl esters in s-SMC exposed to LDL or to granule remnant-bound LDL in the absence (A) or presence (B) of Sandoz compound 58-035. Monolayers of s-SMC were incubated in 1 ml of medium C containing the indicated additions: 100 µg/ml of LDL (b–g), 5 µg/ml of granule remnants (c, f, g), 200 µM oleate–albumin (d, f), or 200 µM linoleate–albumin (e, g) in the absence (panel A) or presence (panel B) of Sandoz compound 58-035 (5 µg/ml). After incubation at 37°C for 60 h, the cellular cholesteryl esters were extracted and assayed by HPLC, as described in Materials and Methods. Values are means ± SD of triplicate incubations.

lated from the aortic intima, the content of linoleic acid, but not oleic acid, is strongly decreased, reflecting oxidation of LDL (49–53).

In atherogenesis, some foam cells also show lysosomal lipid accumulation. A biochemical explanation for this phenomenon was recently put forward, when it was discovered that oxidized LDL is only poorly degraded by lysosomal enzymes (54). From the current findings and the previous observation that mast cells inhibit oxidation of LDL (17), we infer that the foam cells showing lysosomal accumulation of lipids are likely not formed through the action of mast cells. Rather, mast cells in the arterial intima are prone to generate typical foam cells filled with cytoplasmic lipid droplets, especially as mast cell granules also effectively inhibit efflux of cholesterol from the cytoplasmic compartment of foam cells (55, 56).

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